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ORIGINAL ARTICLE

Transglutaminases, involucrin, and loricrin as markers of epidermal differentiation in skin substitutes derived from human sweat gland cells

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Abstract
Background/Purpose In a multi-project research line, we are currently testing whether a morphologically and functionally near normal epidermis can be cultured from human sweat gland (SG) cells and be used as a skin substitute. The present study focuses on the stratum corneum of the epidermis that assumes a vital barrier function for the skin. The main process in the formation of the cornified cell envelope in human epidermis, i.e. crosslinking of proteins and lipids, is catalyzed by several transglutaminases (TG). Therefore, we compared the expression patterns of various TG and their substrates in SG-derived versus keratinocyte-derived epidermal substitutes.
Methods Sweat gland cells, keratinocytes, and fibroblasts were isolated from human skin samples and cultivated separately to generate epidermal substitutes. These were transplanted onto the back of athymic rats. After 2 weeks, the transplants were excised and analyzed histologically as well as by indirect immunofluorescence. We looked at the expression of TG1, 3, 5, and their substrates involucrin and loricrin (=markers of epidermal differentiation) in SG-derived and keratinocyte-derived skin substitutes as well as in normal skin.
Results The SG cell-derived epidermis was near normal anatomically, formed a cornified cell envelope and demonstrated TG1, 3, and 5 as well as involucrin and loricrin expression patterns similar to those found in keratinocyte-derived epidermis and normal control skin.
Conclusion These findings support the thesis that SG cells have the potential to form a near normal stratified epidermal analog that might be used as a skin substitute. The expression of TG1 and 3, not normally expressed in human SG, suggests the presence of re-programmed SG cells and/or stem cells capable of both de novo generating and maintaining an epidermis.

Keywords Tissue engineering · Skin substitutes · Sweat gland · Keratinocyte · Transglutaminase · Involucrin · Loricrin

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Introduction

Burn surgeons as well as plastic and reconstructive surgeons are still facing considerable difficulties when massive skin defects must be covered. No doubt, one of the most recent and promising strategies to overcome this problem is the engineering of skin. Today, cultured epithelial autografts as well as a variety of composite skin substitutes are readily available in many centers. However, the use of cultured skin substitutes is still associated with problems such as poor take, the melting graft phenomenon, and severe scarring, all of which continue to plague patients and clinicians alike [1–6]; and clearly, none of the above laboratory products has the fabulous properties of natural (full thickness) skin grafts. Therefore, our tissue engineering efforts aim at building a skin analog in the laboratory that architecturally and functionally closely approximates normal skin.

One of our projects looks at the question whether SG cells can transdifferentiate into epidermal keratinocytes,

or, whether human SG actually contain stem cells that can produce keratinocytes. We could in fact demonstrate that a stratified and cornified epidermis can be cultured from human SG cells and then be successfully transplanted onto immunoincompetent rats (manuscript in preparation).

One of the crucial tasks of the epidermis is its barrier function, mainly provided by the keratinocytes' capability to form the so-called cornified cell envelope, a protein and lipid containing layer synthesized and deposited directly beneath the plasma membrane. The enzymes TG1, 3, and 5 are the driving force behind the formation of the cornified cell envelope, which is an essential element of epidermal differentiation. They catalyze covalent γ-glutamyl-ε-lysine crosslinks between extracellular matrix proteins [7]. The substrate involucrin is crosslinked by TG and serves as a scaffold for further crosslinkings to form the cornified cell envelope [8–10]. Loricrin, another TG-substrate, is the main component (70%) of the cornified cell envelope and is stored in the granules of the stratum granulosum [11, 12]. TG1 is the only TG anchored in the plasma membrane of keratinocytes. It is expressed in the stratum spinosum and granulosum [13]. TG3 is expressed in the upper stratum granulosum and is found in the cytosol of keratinocytes [14]. TG5 is mainly expressed early during epidermal differentiation and identified in the stratum spinosum and granulosum, to a lesser extent also in the basal layer (perinuclear area of keratinocytes) [15].

The goal of this study is to compare TG1, 3, and 5, as well as their substrates involucrin and loricrin in SG-derived and keratinocyte-derived epidermis. Because these molecules are critically involved in the formation of the cornified cell envelope, they are viewed as markers of epidermal differentiation.

Materials and methods

Human skin specimens

Following the approval by the ethics committee of the Canton of Zurich and after written informed consent by parents and/or patients, skin samples from scalp, abdomen, retroauricular area, or foreskins were obtained from patients aged between 1 and 18 years. The specimens were used for isolation of SG cells, keratinocytes, and fibroblasts, or employed for histological analysis.

Cell cultures

Keratinocytes and fibroblasts were isolated and cultured according to standard protocols as described earlier [16]. For SG cells, we used the following procedure:

The day before isolation, mitomycin-treated or irradiated swiss albino 3T3 mouse fibroblasts (ATCC CCL-92) were seeded at a density of 10,000 per cm^2 in DMEM, 10% calf serum, and 5 μg/ml gentamycin. Immediately before plating the fragments of sweat glands (SGs), the medium was changed to the original Rheinwald and Green medium (RGM) [17, 18].

Skin samples were cut into small pieces ($\sim$6 mm^2), digested in DMEM containing 12 U/ml dispase (Invitrogen, Basel, Switzerland), 2 mg/ml collagenase blend F (Sigma, Buchs, Switzerland), and 38 U/ml collagenase II (Worthington, Bioconcept, Allschwil, Switzerland) for 16 h at 4°C and 4 h at 37°C until the mesenchyme was almost completely digested. Tissue pieces were centrifuged and fat droplets removed with the supernatant. The pellet was re-suspended in 20 ml of DMEM containing threefold concentrated antibiotics (gentamycin 15 μg/ml, penicillin 3,000 U/ml, streptomycin 3 mg/ml, and fungizone 750 ng/ml), 1% FCS, and transferred to a new culture dish. The dermis was then separated from the epidermis and SG fragments were collected using a stereomicroscope and micropipettes. The fragments were washed with culture medium and finally transferred to the culture dish. After 4–5 days, the first outgrowing cells were visible. After 2 weeks, the feeder cells were removed by digestion with 0.1% trypsin, 1 mM EDTA (Invitrogen, Basel, Switzerland) for 2 min at 37°C. The remaining SG cells were washed twice in PBS and detached from the dish with 0.5% trypsin, 5 mM EDTA, for 5 min at 37°C. Trypsin activity was stopped by the addition of 3.75 mg/ml soy bean trypsin inhibitor (Invitrogen, Basel, Switzerland). An almost single cell suspension was achieved by repeated pipetting.

Keratinocytes and SG cells were further expanded during the first passage. Keratinocytes were grown in a serum-free medium (SFM, Invitrogen, containing 0.2 ng/ml EGF and 25 μg/ml bovine pituitary extract). SG cells were maintained in RGM.

Construction of dermo-epidermal substitutes

Substitutes were produced using a previously established transwell system consisting of six-well cell culture inserts with membranes of 3.0-μm pore-size (BD Falcon, Basel, Switzerland) [16]. Briefly, membranes were covered with rat tail collagen type I hydrogels (3.2–3.4 mg/ml, BD Biosciences, Allschwil Switzerland) containing human dermal fibroblasts (passage 1). The collagen matrix was prepared according to the protocol of Costea et al. [18]. These dermal equivalents were grown in DMEM/10% FCS for 5 days. Subsequently, SG cells and keratinocytes were separately seeded onto dermal equivalents at a density of 125×10^3 cells per cm^2 within siliconized polypropylene

rings of 5 mm in diameter to avoid dispersion. After 7 h, the rings were removed, 1 ml RGM was added in the upper chamber, and 2 ml were added to the lower chamber. Triplicate wells were set-up for each dermo-epidermal substitute. The constructs were cultured in RGM. After 4 days, the dermo-epidermal substitutes were raised to the air/liquid interface and cultured for three additional weeks.

Transplantation of cultured dermo-epidermal substitutes

Dermo-epidermal grafts were transplanted onto full thickness skin defects created surgically and encased by polypropylene rings (27-mm diameter) as previously described [16]. Rings were sutured on the back of 10-week-old female athymic Nu/Nu rats. Transplants were covered with a silicon foil. After 14 days, grafts were excised and processed for cryo- and paraffin sections. Isoflurane (Abbott AG, Baar, Switzerland) was used as anesthetic in all procedures.

Histology and immunofluorescence

Excised specimens were embedded in OCT compound (Sakura Finetek/Digitana AG, Horgen, Switzerland) and frozen at −20°C. Cryosections were fixed and permeabilized in acetone for 5 min at −20°C, air-dried, washed three times in PBS, and blocked in PBS, containing 2% BSA (Sigma, Buchs, Switzerland), for 30 min. Incubation with diluted antibodies was performed in blocking buffer for 1 h at room temperature. Slides were washed three times for 5 min in PBS and blocked for additional 15 min before the second antibody was added. If necessary, the same procedure was repeated for the third antibody incubation. Finally, slides were incubated for 5 min in PBS containing 1 μg/ml Hoechst 33342 (Sigma, Buchs, Switzerland), washed twice for 5 min in PBS, and mounted with Dako mounting solution (Dako, Baar, Switzerland) containing 25 mg/ml of DABCO anti-quenching agent (Sigma, Buchs, Switzerland).

Antibodies

For immunofluorescence, the following antibodies were used: From LabVision (P.H.Stehelin&CIE AG, Basel, Switzerland): Involucrin (clone SY5, 1:100); from Abcam (Cambridge, UK): Loricrin (polyclonal, 1:500); from Novus Biologicals (Littleton, CO, USA): transglutaminase 1 (polyclonal, 1:800), transglutaminase 3 (polyclonal, 1:50), transglutaminase 5 (polyclonal, 1:200). As a secondary antibody, we used FITC-conjugated polyclonal goat $F(ab')_2$ fragments directed to mouse immunoglobulins (Dako, Baar, Switzerland). For double IF, some of the primary antibodies were pre-labeled with Alexa 555-conjugated polyclonal goat F(ab′)2 fragments, according to the instructions of the manufacturer (Zenon Mouse IgG Labeling Kit, Molecular Probes, Invitrogen, Basel, Switzerland).

Results

A keratinocyte-derived substitute and an SG-derived substitute are shown in Fig. 1a, b. Basically, in both the substitutes, all epidermal layers are present: the basal layer, 6–8 suprabasal layers, and a well-differentiated stratum corneum. In certain areas, the SG-derived epidermis is less compact or appears somewhat disorganized, while in other areas, there is almost no difference to natural epidermis discernable.

An overview of expression patterns of TG1, 3, 5, as well as involucrin and loricrin in normal skin, SG, SG-derived, and keratinocyte-derived skin substitutes is given in Table 1. In the global picture, SG and keratinocyte-derived skin substitutes phenomenologically demonstrated staining patterns identical to those of normal skin (control). However, there were certain quantitative and site-related differences, which are described below in more detail.

In SG, TG1 (Fig. 1c) and 3 were not expressed, while TG5 (Fig. 1d) expression was found in ductal as well as secretory glandular cells.

In normal skin (=control), TG1 stained positive in the upper stratum spinosum and stratum granulosum with membrane-bound distribution (Fig. 2a). An almost identical pattern was found in SG-derived (here with a slightly more extended weak staining into the lower part of the stratum spinosum) (Fig. 3a) and keratinocyte-derived skin substitutes.

In normal skin, TG3 stained positive in the stratum granulosum (upper layers) and corneum (Fig. 2b). A basically identical picture was obtained in keratinocyte-derived skin. In the SG-derived epidermis, all epidermal layers stained positive for TG3 with a cytoplasmic distribution of the enzyme (Fig. 3b).

In normal skin, TG5 was detected in the stratum spinosum, granulosum, and basale with a perinuclear, intracellular distribution (Fig. 2c). In keratinocyte-derived epidermis, there was a strong positivity for TG5 in all layers with a maximum in the basal layer (perinuclear distribution). The SG-derived substitutes showed an identical TG5 expression pattern as normal skin, with the strongest staining in the basal layer and upper stratum granulosum (Fig. 3c).

In normal skin, involucrin was present in the stratum granulosum and corneum (Fig. 2d). The substitutes derived from keratinocytes and SG cells (Fig. 3d) stained all suprabasal layers positive, whereas the basal layers were

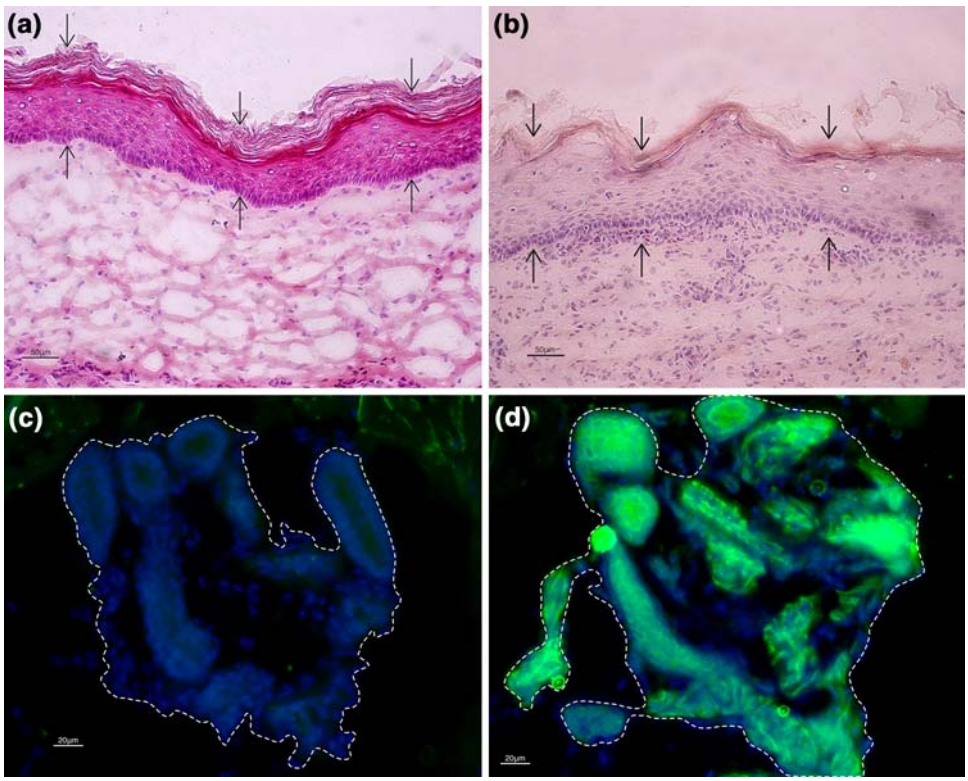

Fig. 1 **a** Epidermal substitute derived from keratinocytes (*between arrows*), 14 days after transplantation on an athymic rat, hematoxylin and eosin, ×40. **b** Epidermal substitute derived from SG cells (*between arrows*), 14 days after transplantation on an athymic rat, hematoxylin and eosin, ×40. **c** Normal human skin. Sweat gland, glandular part (framed by *dashed line*), TG1 negative (*green*), fluorescence microscopy ×40. **d** Normal human skin. Sweat gland, glandular part (framed by *dashed line*), TG5 positive (*green*), fluorescence microscopy ×40

Table 1 Overview of relative expression patterns of markers compared with normal human skin (Nhs)

	Nhs	SGnhs	Esdk	EsdSG
TG1	+	−	+	++
TG3	+	−	+	+++
TG5	+	+	+++	+
Involucrin	+	±[a]	+++	+++
Loricrin	+	−	++	++

Nhs Normal human skin, *SGnhs* sweat gland in normal human skin, *Esdk* epidermal substitute derived from keratinocytes, *EsdSG* epidermal substitute derived from SG cells

[a] Negative in the glandular part, positive in the ductal part of sweat glands

negative. Involucrin distribution was identified along cell membranes with a fine granular staining in the cytoplasm.

In normal skin, loricrin was detected in the upper stratum granulosum and, to a lesser extent, in the stratum corneum. A similar staining is present in the epidermal substitutes. Loricrin stained identical in both substitutes, but it was markedly stronger than in control skin.

Discussion

The focus of the present study was to look at how SG-derived epidermal substitutes compared with keratinocyte-derived epidermal substitutes and normal skin (serving as control) in terms of epidermal differentiation, particularly the formation of the stratum corneum.

As a first step, epidermal substitutes using SG cells or keratinocytes were grown in culture, then transplanted onto immunoincompetent rats, harvested 2 weeks thereafter, and finally analyzed histologically with particular regard to established markers for epidermal differentiation (TG1, 3, 5 as well as involucrin and loricrin).

In general, both SG and keratinocyte-derived skin substitutes exhibited a near normal epidermal architecture with a basal layer, a correct number of suprabasal cell layers, and a well-developed stratum corneum. The fact that both substitutes survived for 2 weeks on an animal, moreover, indicates that the near natural epidermal anatomy also provided sufficient function, particularly barrier function.

However, the key findings of this experiment are the astoundingly similar expression patterns of both substitutes and normal skin with regard to the epidermal differentiation markers tested. The following points deserve a comment:

First, TG1, 3, and 5, all present in normal skin, were also identified in both epidermal substitutes. Although this is rather to be expected for keratinocyte-derived skin, it represents a striking finding for the SG-derived substitute, since in human SG, only TG5 can be identified. It is reasonable to assume that SG cells cultured to form an epidermal equivalent may undergo a re-programming process

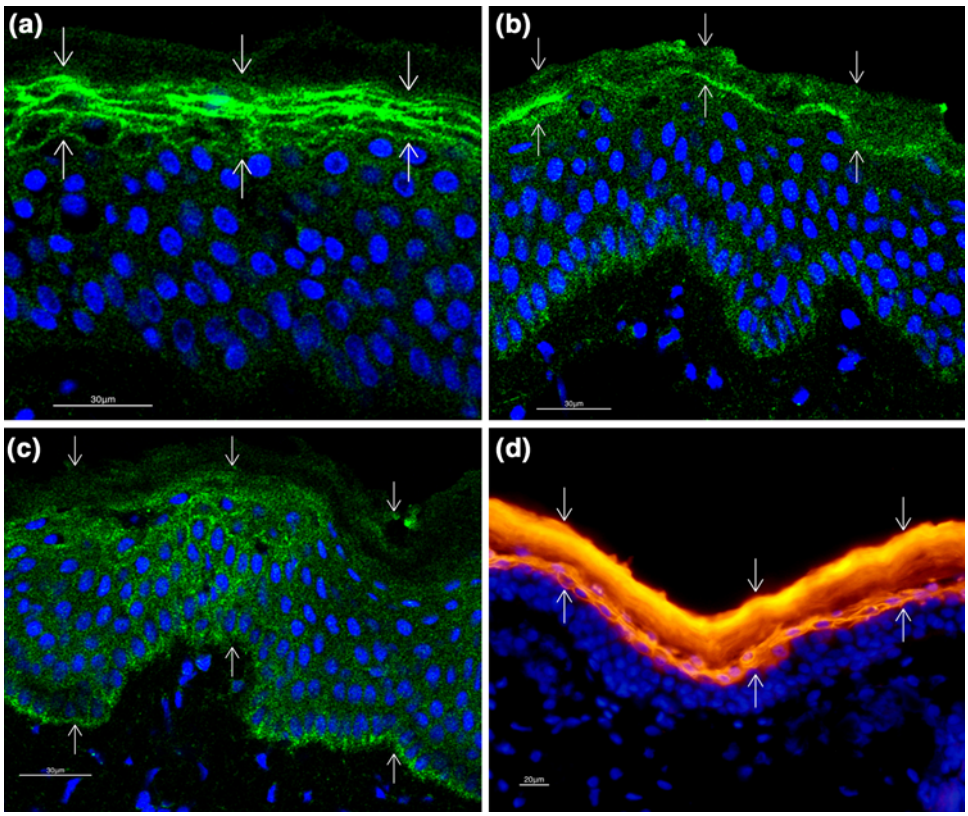

Fig. 2 **a** Normal human skin. Stratum spinosum and granulosum (between *arrows*), TG1 positive (*green*), confocal microscopy ×40. **b** Normal human skin. Stratum granulosum and corneum (between *arrows*), TG3 positive (*green*), confocal microscopy ×40. The remaining areas with seemingly positive signals are background signals. **c** Normal human skin. Stratum basale, spinosum, and granulosum (between *arrows*), TG5 positive (*green*), confocal microscopy ×40. **d** Normal human skin. Stratum granulosum and corneum (between *arrows*), involucrin positive (*red*), fluorescence microscopy ×40

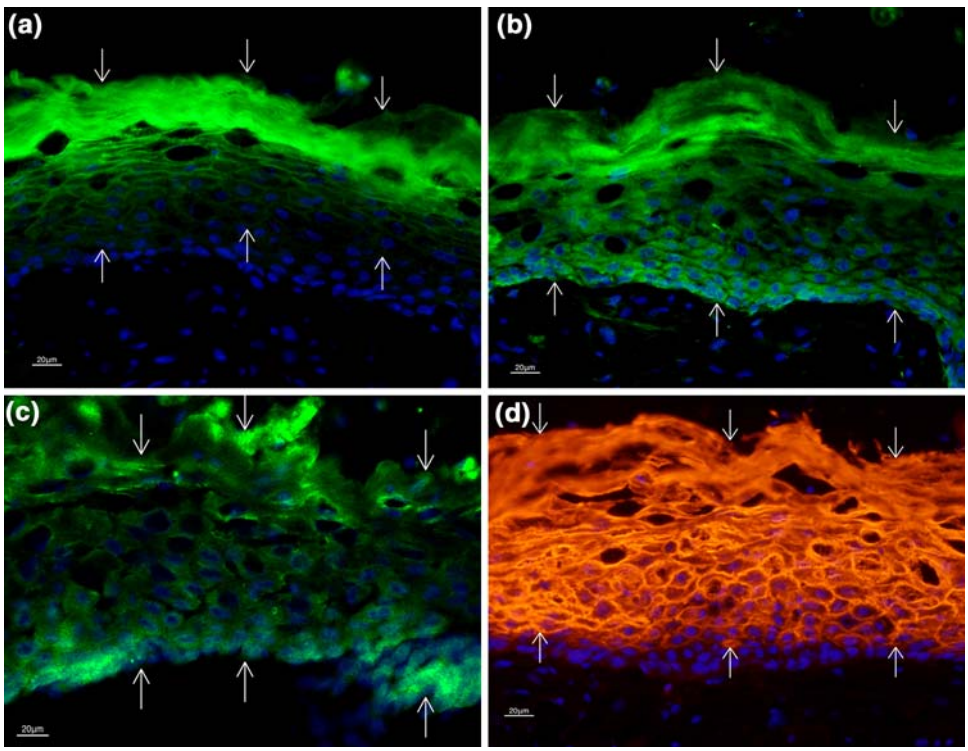

Fig. 3 **a** Epidermal substitute derived from SG cells. Stratum spinosum and granulosum (between *arrows*), TG1 positive (*green*), fluorescence microscopy ×40. **b** Epidermal substitute derived from SG cells. Stratum basale, spinosum, granulosum, and corneum (between *arrows*), TG3 positive (*green*), fluorescence microscopy ×40. **c** Epidermal substitute derived from SG cells. Stratum basale, spinosum, and granulosum (between *arrows*), TG5 positive (*green*), fluorescence microscopy ×40. **d** Epidermal substitute derived from SG cells. Stratum spinosum, granulosum, and corneum (between *arrows*), involucrin positive (*red*), fluorescence microscopy ×40

allowing TG1 and 3 expressions again. Alternatively, stem cells, present in the culture, might promote expression of those enzymes crucial for the formation of the cornified cell envelope. It is noteworthy in this context that Brouard has suggested that human SGs might harbor epidermal stem cells [19].

The second aspect regards some distinct TG staining differences between the skin substitutes and control skin. Although TG1 and 5 expression was virtually identical in SG derived and control skin, TG3 stained positive in all layers of SG-derived skin, but only in the stratum granulosum of normal skin. In contrast, in keratinocyte-derived skin the TG1 and 3 patterns were the same as in control skin, while TG5 stained positive in all layers, whereas it was only found in the stratum spinosum and granulosum of control skin. These differences can be viewed as a (partially) delayed epidermal differentiation. This explanation appears particularly plausible for SG cell-derived skin, as here, a probably intricate transition from ductal or secretory glandular cells to interfollicular epidermal keratinocytes must be accomplished. The literature does not offer any direct corroboration for our thesis. On the other hand, Inada et al. [20] showed that under wound healing conditions, TG1 was initially expressed in all suprabasal layers, whereas later on, in a homeostatic situation, TG1 was only found in the stratum spinosum and granulosum. Therefore, it is conceivable that, analogously, TG3 (in SG-derived skin) and TG5 (in keratinocyte-derived skin) are upregulated early after transplantation. Of note, wound healing studies investigating regulation and expression of TG3 and 5 do not exist.

Thirdly, we showed that involucrin and loricrin were expressed in both skin substitutes (of note, these molecules are not identifiable in SG). This is an important confirmation that epidermal differentiation also takes place with regard to TG substrates, which are the key components of the cornified cell envelope indispensable for epidermal barrier function [7–12]. Here, again, we found an impressive overexpression of involucrin that parallels the one identified on the enzyme level, indicating that homeostasis is not yet reached, i.e. terminal differentiation is not yet accomplished. Interestingly, Stark et al. [21] reports that involucrin is often upregulated in vitro. This might be an alternative explication for the mentioned overexpression. In contrast, loricrin was similarly expressed in both substitutes and control skin.

In conclusion, we demonstrated that an epidermis can be cultured from human SG cells and be successfully transplanted as a skin substitute. The near normal expression of epidermal differentiation markers, especially TG1 and 3, not normally expressed in human SG, suggests the presence of re-programmed SG cells, and or stem cells capable of both de novo generating and maintaining an epidermis.

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Conflict of interest statement The authors declare that none of them have any conflict of interest.

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